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MPP8 and SIRT1 Crosstalk in E-cadherin Gene Silencing and Epithelial-Mesenchymal Transition

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Esther Schnapp

1st Editorial Decision 21 November 2014

Thank you for the submission of your research manuscript to EMBO reports. We have now received the enclosed referee reports on it.

As you will see, the referees acknowledge that the interaction between MPP8 and SIRT1 in the silencing of the E-cadherin gene is potentially interesting. However, they also point out inconsistencies with published data and technical issues (low data quality, insufficient numbers of cells) that would need to be addressed for publication of the manuscript by EMBO reports. While referee 2 is rather critical in her/his report, s/he mentions in the referee cross-comments that her/his most important concern is the unclear relative roles of MPP8 and ZEB1 in SIRT1 recruitment to the E-cadherin promoter and the roles of H3K9ac versus H4K16ac in E-cadherin silencing. Referee 3 agrees in her/his cross-comments with referee 1 that the number of cells used to generate the data shown in figures 6B and C is too low. S/he also agrees with referee 2 that the role of H4K16ac in E-cadherin silencing remains unclear, and that the discrepancies of the current data with previously published ones should be addressed. S/he suggests that it could be examined whether the roles of MPP8 and ZEB1 are independent of each other or additive.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. The most important concerns that need to be addressed experimentally are mentioned above and also include all points that relate to data quality and missing controls, but please address all

referee concerns in a point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS:

Referee #1:

This study illustrated a novel mechanism whereby class III HDAC SIRT1 and MPP8 reciprocally promotes each other's function and coordinate for epithelial gene silencing and EMT. The authors have demonstrated that MPP8 cooperates with SIRT1 through their physical interactions, and SIRT1 antagonizes PCAF-catalyzed MPP8-K439 acetylation to protect MPP8 from ubiquitin-proteasome mediated proteolysis. Additionally the authors found that MPP8 recruits SIRT1 for H4K16 deacetylation after binding to methyl-H3K9 on target promoters. Finally, the authors demonstrate that disabling either MPP8 methyl-H3K9 binding or SIRT1 interaction de-represses E-cadherin and imitates EMT phenotypes caused by knockdown of MPP8 or SIRT1 in prostate cancer cells. These findings are very important and provide mechanistic insights into the role of MMP8 and SIRT1 cooperation in regulating E-cadherin gene silencing and EMT.

Weaknesses:
The data in Figure 6B and 6C are not convincing. Figure 6B is to show the E-cadherin localization in the cell-cell contact after mesenchymal to epithelial transition, however, figure 6B right now shows very limited E-cadherin localization in the cell-cell contact; the immunestaining quality also needs to improve. This raises a concern to whether there is rearrangement of E-cadherin localization in the cell-cell contact and whether there is an obvious EMT morphology change.
Additionally, both Figure 6B and 6C only included 2-10 cell, to provide more convincing data showing the E-cadherin localization and EMT morphology changes, a population of cells is needed.

Minor Weaknesses:
1. Which concentration or treatment condition of SIRT1 inhibitors such as E-527 was used in this study was not indicated in the figure legends or in the methods section.

2. The western quality need to be improved in figure 6A. The fibronectin band is not clear.

Referee #2:

In this manuscript, the authors describe the functional interplay between the H3K9methyl-binding protein MPP8 and the NAD-dependent deacetylase SIRT1 in the silencing of the E-cadherin gene. The authors demonstrate that both proteins interact, characterize the domains involved in the interaction, and determine that MPP8 recruits SIRT1 to the E-cadherin gene. They also demonstrate that upon interaction, SIRT1 deacetylates MPP8 in the residue K439Ac and stabilizes MPP8 protein levels by inhibiting his poly-ubiquitination and subsequent degradation by the proteasome. Additionally, they also identify PCAF as the HAT that catalyzes this mark. The authors demonstrate by ChIP experiments that upon recruitment of SIRT1, the levels of H4K16Ac in E-cadherin promoter decrease in a SIRT1-dependent manner. Surprisingly, loss of SIRT1 or MPP8 does not have any effect on H3K9 acetylation or methylation. Finally, they also show that loss of either MPP8 or SIRT1 induces re-expression of E-cadherin gene and recover certain epithelial features such as a cell shape, cell-cell contacts, and a decrease in the capacity of migration or invasion.

The authors have convincingly demonstrated the interplay between both factors in the silencing of the E-cadherin gene and the involvement of both in EMT. However, the manuscript has several major issues. The main one is, in my opinion, the novelty of the data presented. The interplay between both factors is completely new, including the identification of K439Ac. But that´s about it. The role of both SIRT1 and MPP8 in E-cadherin expression and their impact in EMT was previously demonstrated (Pruitt et al, 2006, Byles et al 2012 and Kokura et al, 2010). If the authors would have tried to connect this interplay with the rest of the factors and marks previously described in the E-cadherin promoter, this would have been an important advance in understanding the sequential order of events that trigger silencing in the E-cadherin gene. For instance, what is the role of this H4K16Ac deacetylation? Where does it exactly take place in the E-cadherin gene? Only in the promoter? How all of this relates to the link between MPP8 and DNA methylation in E-cadherin gene described by the authors previously (Kokura et 2010) given the direct link described for SIRT1 and DNMTs (Espada et al, 2007)? Addressing any of these issues would improve significantly the work.

This issue is directly linked to the second major issue, which is certain inconsistency between the data presented and previously published data. In 2012, Byles et al reported in Oncogene that SIRT1 interacts with the transcription factor ZEB1 and is recruited to the E-cadherin promoter by ZEB1 to deacetylate H3K9Ac and induce silencing. How do the authors reconcile this evidence with their data? Is possible that both ZEB1 and MPP8 interact? Considering that both MPP8 and ZEB1 have been shown to interact with G9a/GLP (in the CtBP1 repressor complex, Shi et al 2013, and Kokura et al 2010). Why in this manuscript there is no effect over H3K9 but there was a clear effect in the Oncogene article? Or alternatively, is mechanistically different the establishment of the E-Cadherin gene silencing from the epigenetic maintenance of this silencing? The authors should address these issues.

A third issue, is related to the model of how SIRT1 protects MPP8. Why deacetylation of K439Ac is required to inhibit poly-ubiquitination of MPP8? Analyzing what they show in the manuscript, does not seem that deacetylation is required for interaction with SIRT1 (WT or H363Y mutant) or to allow another big modification such as sumoylation or non-degrading ubiquitination. Do the authors have any explanation?

MINOR ISSUES

1) Page 13. Lane 4 from bottom. I think "SNAIl" is "SNAIL"

2) Page 15. Lane 3rd lane from top, 2nd paragraph. "SRT1" should be replaced by "SIRT1"

Referee #3:

In this manuscript, Sun and co-workers showed that MPP8 and SIRT1 directly interact on E-cadherin promoter and suppress the expression of E-cadherin. This is mediated by MPP8 binding to methyl-H3K9 and recruitment of SIRT1 via the direct interaction of MPP8 and SIRT1. SIRT1 de-acetylate H4K16 rather than H3K9 to suppress E-cadherin. The authors have nicely mapped the binding regions of the MPP8 and SIRT1 and provided well-controlled experiments for the interaction. Additionally, they also showed that class III acetyltransferase PCAF can acetylate MPP8 on K439 to mediate the degradation of MPP8, which can be counteracted by SIRT1. This study provided solid data for the novel interaction of MPP8 and SIRT1, the specific function of this interaction on the epigenetic regulation of E-cadherin promoter, as well as additional new data on the protein regulation of MPP8 by counteracting epigenetic regulators. This is a complete and solid study that is suitable for publication in EMBO Reports. I have only a few minor comments:

1. From the effect of MPP8-KD and rescue experiment on FN1 level, it indicates that MPP8 and SIRT1 interaction is important for the FN1 level as well. It may require a different mechanism of regulation from the SIRT1-H4K16 acetylation as depicted here for E-cadherin. For example, the protein stability of MPP8 and interaction with other protein complexes. This should be reflected in

the discussion.
2. EMT regulation involves multiple epithelial and mesenchymal markers. This study is particularly focused on E-cadherin. Thus, to extrapolate and claim this MPP8-SIRT1-H4K16ac regulation on EMT, a larger cohort of the markers is required. The authors should tone down the conclusion (or title) of the study accordingly.
3. In Figure 5C, to rescue the effect, the last set of condition should be HA-Mpp8-K439R+Flag-PCAF+myc-SIRT1-H363Y mutant rather than WT SIRT1.
4. The important ChIP experiment validating the mechanism of regulation on E-cadherin (Fig. 7) should be repeated in another cell line MDA-MB-231.
5. On page 5, first paragraph, "As shown in Fig. 1E, E-cadherin mRNA level increased 3-8 folds upon EX-527 treatment ...". It should be Fig. 1D rather than 1E.
6. Figure 5A, the Western blot should be labeled.

1st Revision - authors' response 28 February 2015

Response to the reviewers' comments

We would like to thank the reviewers again for their thoughtful comments, which have allowed us to improve our manuscript significantly. We hope that all reviewers will find that we have addressed their concerns satisfactorily.

Referee #1:

1) The data in Figure 6B and 6C are not convincing. Figure 6B is to show the E-cadherin localization in the cell-cell contact after mesenchymal to epithelial transition, however, figure 6B right now shows very limited E-cadherin localization in the cell-cell contact; the immunestaining quality also needs to improve. This raises a concern to whether there is rearrangement of E-cadherin localization in the cell-cell contact and whether there is an obvious EMT morphology change. Additionally, both Figure 6B and 6C only included 2-10 cell, to provide more convincing data showing the E-cadherin localization and EMT morphology changes, a population of cells is needed.

Following the reviewer's suggestion, we redid immunostaining and differential interference contrast microscopy analyses of different MPP8/SIRT1 knockdown and rescue PC3 cells and took pictures at 10x or 20x magnifications of the objective lens. As indicated in the new Fig4B and 4C, E-cadherin localization in the cell-cell contact and cell morphologic changes can be clearly observed in a large population of cells, suggesting the obvious EMT phenotype changes upon disruption of SIRT1, MPP8 or their interaction.

2) Which concentration or treatment condition of SIRT1 inhibitors such as E-527 was used in this study was not indicated in the figure legends or in the methods section.

The concentration of EX-527 we used is 1 μM and we have included the concentration of EX-527 as well as other inhibitors in the figure legends of the revised manuscript.

3) The western quality need to be improved in figure 6A. The fibronectin band is not clear.

Following the reviewer's suggestion, we redid fibronectin western blot analysis of different knock-down and rescue PC3 cells. The new results (Fig.4A) show a clear fibronectin protein band.

Referee #2:

1) If the authors would have tried to connect this interplay with the rest of the factors and marks previously described in the E-cadherin promoter, this would have been an important advance in understanding the sequential order of events that trigger silencing in the E-cadherin gene. For instance, what is the role of this H4K16Ac deacetylation? Where does it exactly take place in the

E-cadherin gene? Only in the promoter? How all of this relates to the link between MPP8 and DNA methylation in E-cadherin gene described by the authors previously (Kokura et 2010) given the direct link described for SIRT1 and DNMTs (Espada et al, 2007)? Addressing any of these issues would improve significantly the work.

The reviewer raised a great question about how different epigenetic modifications, including different histone deacetylations and DNA methylation cooperate for E-cadherin silencing. We have previously revealed that MPP8 binds to H3K9me marks on promoter and recruits DNMT3A for DNA methylation (Kokura et al 2010). In this study, we further demonstrate that MPP8 promoter binding is also a prerequisite for SIRT1 targeting and H4K16 deacetylation. Our results indicate that MPP8-Δ112-225 mutant (deficient in SIRT1 interaction) binds to E-cadherin promoter similarly to MPP8-wt (dataset4 in Fig.5A, S2B). However, it only moderately represses gene expression (Fig.4A, S2A). Therefore, SIRT1 recruitment and stepwise H4K16 deacetylation are critical for MPP8-mediated repression.

Given that MPP8 binds to both H3K9me and DNMT3A in the same methyl-lysine dependent manner (Kokura et al 2010, Chang 2011), we believe that MPP8 recruits SIRT1 and DNMT3A independently through different domains. Moreover, the findings that SIRT1 not only interacts with DNMT1 (Espada et al, 2007), but also deacetylates DNMT1 to increase its enzymatic activity, suggesting that SIRT1 may also facilitate DNA methylation. We have discussed these possibilities in the revised manuscript.

Several genome studies demonstrate that H4K16 acetylation is enriched around the transcriptional start site of active genes in CD4+ Tcells and ESCs (Nat Genet. 2008 40:897, Genome Res. 2013, 23:2053). Consistently, we detected significantly increased H4K16 acetylation in promoter of two SIRT1 target genes, E-cadherin and CRBP1. Intriguingly, a proportion H4K16 acetylation fell into enhancer regions (Genome Res. 2013, 23:2053), indicating that it has multiple functions in transcription activation. We are very interested in carrying out following study to further understand the functional significance of our working model on genome-wide.

2) This issue is directly linked to the second major issue, which is certain inconsistency between the data presented and previously published data. In 2012, Byles et al reported in Oncogene that SIRT1 interacts with the transcription factor ZEB1 and is recruited to the E-cadherin promoter by ZEB1 to deacetylate H3K9Ac and induce silencing. How do the authors reconcile this evidence with their data? Is possible that both ZEB1 and MPP8 interact? Considering that both MPP8 and ZEB1 have been shown to interact with G9a/GLP (in the CtBP1 repressor complex, Shi et al 2013, and Kokura et al 2010). Why in this manuscript there is no effect over H3K9 but there was a clear effect in the Oncogene article? Or alternatively, is mechanistically different the establishment of the E-Cadherin gene silencing from the epigenetic maintenance of this silencing? The authors should address these issues.

The reviewer raised another great point about how epigenetic machineries cooperate with transcription factors. Following the reviewer's suggestion, we determined whether MPP8 interacts with ZEB1. As indicated in Fig.5C&5D, our IP-western results demonstrate that MPP8 not only specifically interacts with ZEB1, but also significantly facilitates ZEB1-SIRT1 interaction. We further confirmed these interactions by endogenous IP-western analysis. These results suggest a possibility that both SIRT1 and MPP8 are recruited by ZEB1 to target promoters while MPP8 also contributes to SIRT1 recruitment by bridging or stabilizing ZEB1-SIRT1 interaction. As the reviewer pointed out, ZEB1 was co-purified with G9a/GLP in CtBP1 repressor complex, suggesting that the mechanistic detail underlying these recruitments is complicated. We are now doing the follow up study to dissect MPP8-ZEB1 interaction and hope to develop an independent story in the near future.

Our ChIP analyses reveal that H4K16 acetylation increases significantly while H3K9 acetylation only increases slightly on both E-cadherin and CRBP1 promoter upon knockdown of SIRT1 or disruption its interaction with MPP8 (Fig.5, S4. S5). These results are consistent with the previous mechanistic study showing that SIRT1 preferentially deacetylates H4K16 over other histone lysine residues including H3K9 at the physiological concentration (Mol Cell. 2004, 16: 93). In a recent Oncogene article, Byles et al demonstrated that knockdown of ZEB1 results in a significant increase of H3K9 acetylation on E-cadherin promoter. However, they did not test whether knockdown of

SIRT1 has the similar effect. Another recent report (Gut. 2012, 61:439) demonstrate that knockdown of ZEB1 delocalizes HDAC1/2 from E-cadherin promoter accompanied by increased histone acetylation, suggesting that ZEB1 recruits multiple HDACs to deacetylate different histone lysine residues. As ZEB1 was co-purified with HDAC1/2 in CtBP1 complex and knockdown of CtBP1 increases H3K9 acetylation on E-cadherin promoter, we predict that ZEB1 knockdown-induced increase on H3K9 acetylation on E-cadherin promoter could be mainly attributed to the delocalization of CtBP1 complex (or other HDAC-containing complex recruited by ZEB1).

Furthermore, we also determined the role of H3K9 deacetylation in our model as H3K9 acetylation has also been associated with E-cadherin expression in multiple studies. After TSA treatment, we detected a dosage dependent increase of E-cadherin expression in both control and SIRT1-KD PC3 cells. When normalized to control treatment, E-cadherin expression displayed the similar up-regulation pattern in both cells (Fig.S5A-C). Therefore, we believe that TSA treatment and SIRT1 knockdown induce E-cadherin expression in a non-overlapping manner. We also carried out ChIP-qPCR analysis and our results reveal that TSA treatment significantly increased H3K9 acetylation on E-cadherin promoter in both control and SIRT1-KD PC3 cells, but only moderately affected H4K16 acetylation (Fig.S4D-E). These results together suggest that both H3K9 and H4K16 deacetylation play an important role in E-cadherin gene silencing but they obviously contribute to different repression pathways.

3) Page 13. Lane 4 from bottom. I think "SNAIl" is "SNAIL"
4) Page 15. Lane 3rd lane from top, 2nd paragraph. "SRT1" should be replaced by "SIRT1"

We have corrected these typos in the text.

Referee #3:

1) From the effect of MPP8-KD and rescue experiment on FN1 level, it indicates that MPP8 and SIRT1 interaction is important for the FN1 level as well. It may require a different mechanism of regulation from the SIRT1-H4K16 acetylation as depicted here for E-cadherin. For example, the protein stability of MPP8 and interaction with other protein complexes. This should be reflected in the discussion.

The reviewer raised a very interesting point. Given that we have previously demonstrated that MPP8 does not target fibronectin promoter (Kokura et al 2010), we believe that it is an indirect effect. We have also included this in the discussion.

2) EMT regulation involves multiple epithelial and mesenchymal markers. This study is particularly focused on E-cadherin. Thus, to extrapolate and claim this MPP8-SIRT1-H4K16ac regulation on EMT, a larger cohort of the markers is required. The authors should tone down the conclusion (or title) of the study accordingly.

Following the reviewer's suggestion, we have toned down the conclusion in the text of the revision.

3) In Figure 5C, to rescue the effect, the last set of condition should be HA-Mpp8-K439R+Flag-PCAF+myc-SIRT1-H363Y mutant rather than WT SIRT1.

We really appreciate that this reviewer pointed out this mistake. The last set of Fig.5C (new Fig.3C) was mislabeled during our figure preparation and it should be SIRT1-H363Y mutant instead of SIRT1-wt. We have corrected this mistake in both figure and text of the revised manuscript.

4. The important ChIP experiment validating the mechanism of regulation on E-cadherin (Fig. 7) should be repeated in another cell line MDA-MB-231.

Following the reviewer's suggestion, we generated the same set of stable knockdown and rescue MDA-MB-231 cells and carried out the gene expression and ChIP-qPCR analysis. As indicated in Fig.S2, we detected the similar gene expression and localization changes in MDA-MB-231 cells. These results further validated the regulation mechanism we identified.

5. On page 5, first paragraph, "As shown in Fig. 1E, E-cadherin mRNA level increased 3-8 folds upon EX-527 treatment ...". It should be Fig. 1D rather than 1E.

We have corrected these mistakes.

6. Figure 5A, the Western blot should be labeled.

We have labeled the western blot in Fig.5A (new Fig.3A).

2nd Editorial Decision 12 March 2015

Thank you for the submission of your revised manuscript to our journal. We have now received the reports from the referees that were asked to assess it, and both support publication of the study now, which we can therefore in principle accept.

Referee 2 only asks you to include ZEB1 in the model in figure 5. I would like to add that the number of independent experiments, n, is not clearly mentioned in several figure legends. Figure legend 1B says "3 reactions", 4D "3 independent samples", what does this mean exactly? Are these 3 independent experiments, or 3 parallel, technical replicates of 1 experiment? Please modify the text to make this clear. If n<3 no error bars can be shown. The quantification in figure 3A needs to be mentioned in the figure legend, including n and the definition of the error bars. Legend 1B also needs to specify the error bars, and legends 5, S5 and S2 need to mention the test used to calculate p-values.

The overall character count is also still rather high, and it would be good if you could shorten the text/discussion further, if possible.

I also would like to suggest a few changes to the abstract, as follows:

As a critical developmental process, epithelial-mesenchymal transition (EMT) involves complex transcriptional reprogramming and has been closely linked to malignant progression. Although various epigenetic modifications, such as histone deacetylation and H3K9 methylation have been implicated in this process, how they are coordinated remains elusive. We recently revealed that MPP8 couples H3K9 methylation and DNA methylation for E-cadherin gene silencing and promotes tumor cell migration, invasion and EMT. Here, we show that MPP8 cooperates with the class III HDAC SIRT1 in this process through their physical interaction. SIRT1 antagonizes PCAF-catalyzed MPP8-K439 acetylation to protect MPP8 from ubiquitin-proteasome mediated proteolysis. Conversely, MPP8 recruits SIRT1 for H4K16 deacetylation after binding to methyl-H3K9 on target promoters. Consequently, disabling either MPP8 methyl-H3K9 binding or SIRT1 interaction de-represses E-cadherin and reduces EMT phenotypes, as does knockdown of MPP8 or SIRT1 in prostate cancer cells [OK? I think that in the absence of functional MMP8 no EMT occurs]. These results illustrate how SIRT1 and MPP8 reciprocally promote each other's function and coordinate epithelial gene silencing and EMT.

Please let me know whether you agree with these changes.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS:

Referee #2:

In the new version of the manuscript, the authors have answered my previous concerns satisfactorily. Their changes to the previous version, including new data and discussion, have

increased significantly the overall quality and novelty of the work. The link to ZEB1 (added in Figures 5C and D) and the new data with TSA (in suppl Figure 5), clearly delimits the contribution of SIRT1 to H4K16ac vs H3K9ac in E-cadherin repression and integrates this data more convincingly in the body of evidence previously reported. My only comment would be that considering the new data added in the new version of the manuscript, I would recommend that the model in figure 5G also includes ZEB1.

2nd Revision - authors' response 16 March 2015

Thank you very much for accepting our manuscript entitled "MPP8 and SIRT1 Crosstalk in E-cadherin Gene Silencing and Epithelial-Mesenchymal Transition" for publication in *EMBO Reports*. We are very happy that we have addressed all reviewers' concerns. Following the suggestions from you and the reviewers, we have made following modifications in the enclosed manuscript.

1) Included ZEB1 in our working model (Fig.5G). We also changed the model figure to grayscale.
2) Removed error bars from Fig.1B, Fig.3A, 4D, Fig.S5B and S5C.
3) Modified figure legends correspondingly, and included method used for *p*-value calculation.
4) Shortened the text and final character count is 32,106 (including space, excluding references).
5) Modified the abstract according to your kind suggestions.
6) Moved the general cell culture info and primer sequences to supplemental materials.

Again, I would like to thank you and three reviewers for all your kind suggestions and generous help, which helped us tremendously to improve the quality of our manuscript. We are very fortunate to have you as our editor this time and I am looking forward to having another opportunity to work with you again in the near future. Please feel free to let me know if you have any question or concern.

3rd Editorial Decision 17 March 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

